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ORIGINAL ARTICLE

Tetrahydrocurcumin Add-On therapy to losartan in a rat model of diabetic nephropathy decreases blood pressure and markers of kidney injury

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Abstract

Tetrahydrocurcumin (THC), a principal metabolite of curcumin, was tested in a rat model of type 2 diabetes mellitus. THC was administered via daily oral gavage with the lipid carrier polyenylphosphatidylcholine (PPC) as add-on therapy to losartan (angiotensin receptor blocker) to examine effects on kidney oxidative stress and fibrosis. A combination of unilateral nephrectomy, high-fat diet and low-dose streptozotocin was used to induce diabetic nephropathy in male Sprague–Dawley rats. Animals with fasting blood glucose >200 mg/dL were randomized to PPC, losartan, THC + PPC or THC + PPC + losartan. Untreated chronic kidney disease (CKD) animals had proteinuria, decreased creatinine clearance, and evidence of kidney fibrosis on histology. THC + PPC + losartan treatment significantly lowered blood pressure concurrent with increased messenger RNA levels of antioxidant copper-zinc-superoxide dismutase and decreased protein kinase C-α, kidney injury molecule-1 and type I collagen in the kidneys; there was decreased albuminuria and a trend for increased creatinine clearance compared to untreated CKD rats. There was decreased fibrosis on kidney histology in PPC-only and THC-treated CKD rats. Plasma levels of kidney injury molecule-1 were decreased in THC + PPC + losartan animals. In summary, add-on THC to losartan therapy improved antioxidant levels and decreased fibrosis in the kidneys, and lowered blood pressure in diabetic CKD rats.

KEYWORDS
animal models, chronic kidney diseases, diabetic nephropathy, losartan, proteinuria, tetrahydrocurcumin

Abbreviations: CKD, chronic kidney disease; col1a1, collagen type I, alpha 1; CTGF, connective tissue growth factor; CuZnSOD, copper-zinc superoxide dismutase; DM, Diabetes mellitus; Dnm1l, Drp1, dynamin-related protein 1; Fis1, mitochondrial fission 1 protein; Fn1, fibronectin-1; Gpx1, glutathione peroxidase; Havcr1, hepatitis A virus cellular receptor 1; Hmox1, heme oxygenase 1; Keap1, Kelch Like ECH Associated Protein 1; KIM-1, kidney injury molecule-1; MFF, mitochondrial fission factor; Nfe2l2, Nrf2, nuclear factor erythroid 2-related factor 2; PAS, periodic acid Schiff; PPC, polyenylphosphatidylcholine; PRKCA, protein kinase C-α; SGLT2, sodium-glucose co-transporter-2; Sirt1, sirtuin 1 (Sirt1, Rn01428096_m1); Smad 3, SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic") family member 3; TGF-β1, transforming growth factor-β1; THC, tetrahydrocurcumin.

Mahyar Khazaeli and Ane C.F. Nunes contributed equally to the manuscript.

1 | INTRODUCTION

Diabetes mellitus (DM) is a major public health issue with a global prevalence of >450 million adults, which is anticipated to climb to 700 million people by 2045.[1] Diabetic kidney disease affects 25% of patients with DM, and is the leading cause of end-stage kidney failure in the developed world.[2] While there have been significant advances in antidiabetic medications, most recently with the sodium-glucose co-transporter-2 (SGLT2) inhibitors, it is estimated that 80% of the world population utilizes complementary and alternative medicine and this is a promising area for DM therapy.[3] Curcumin is one complementary medicine compound that is being explored in various diseases ranging from cancer and immunodeficiencies to DM and hypertension.[4,5]

Curcumin or diferuloylmethane, the major active component of turmeric, is extracted from the dry rhizome of *Curcuma longa* Linn (Zingiberaceae). In the intestine, curcumin is metabolized into curcumin glucuronide, curcumin sulphate, tetrahydrocurcumin (THC), hexahydrocurcumin and octahydrocurcumin.[6–8] The bioavailability of curcumin is low due to poor intestinal absorption, and rapid hepatic metabolism and systemic elimination[4,9] with 75% of ingested curcumin being excreted in the feces.[10] A mouse study demonstrated that 99% of curcumin in plasma was present as glucuronide conjugates and THC[11] (Figure 1).

In models of acute and chronic kidney damage, THC has been shown to exert beneficial antioxidative effects. Via inhibition of lipid peroxidation and upregulation of antioxidant catalytic activity, THC prevented ferric nitrilotriacetate and chloroquine-induced oxidative kidney injury.[12,13] In diabetic rat models, curcumin and THC have been shown to decrease tissue oxidative stress and dyslipidemia, decrease albuminuria, and improve plasma insulin levels.[12,14,15] THC has better enteric absorption than curcumin however bioavailability remains an issue due to its low water solubility.[9] Administration of curcumin with a lipid carrier such as polyenylphosphatidylcholine (PPC) has been shown to increase plasma levels five-fold in rats.[16] Aside from its utility as a lipid carrier, PPC itself has shown benefit in animal DM models via exerting cytoprotective effects on liver and pancreatic β-cells.[17,18]

The renin-angiotensin-aldosterone system (RAAS) has a central role in the pathogenesis of diabetic kidney disease, and RAAS blockade is a mainstay of renoprotection in DM patients with albuminuria.[19,20] Proteinuria is an established independent predictor of increased cardiovascular morbidity and mortality.[21] In animal models of diabetic nephropathy, the angiotensin-II-receptor blocker losartan significantly decreased proteinuria and ameliorated glomerulopathy.[22] However, RAAS blockade does not decrease urinary protein to normal levels; in the RENAAL (Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan) study of patients with type 2 DM and proteinuria, losartan was associated with a 34.3% decrease in proteinuria when compared to placebo.[23] Thus, emerging therapies should be tested in addition to RAAS blockade to evaluate for incremental benefit.

We previously studied THC in 5/6 nephrectomized rats whereby 1% THC with PPC in the diet improved expression of antioxidant enzymes in the remnant kidney, decreased fibrosis and ameliorated proteinuria and hypertension.[24] The current study investigates THC administered with the lipid carrier PPC with/without losartan, in a rat model of type 2 diabetic nephropathy to characterize effects on kidney outcomes. In addition to blood pressure (BP) and proteinuria,

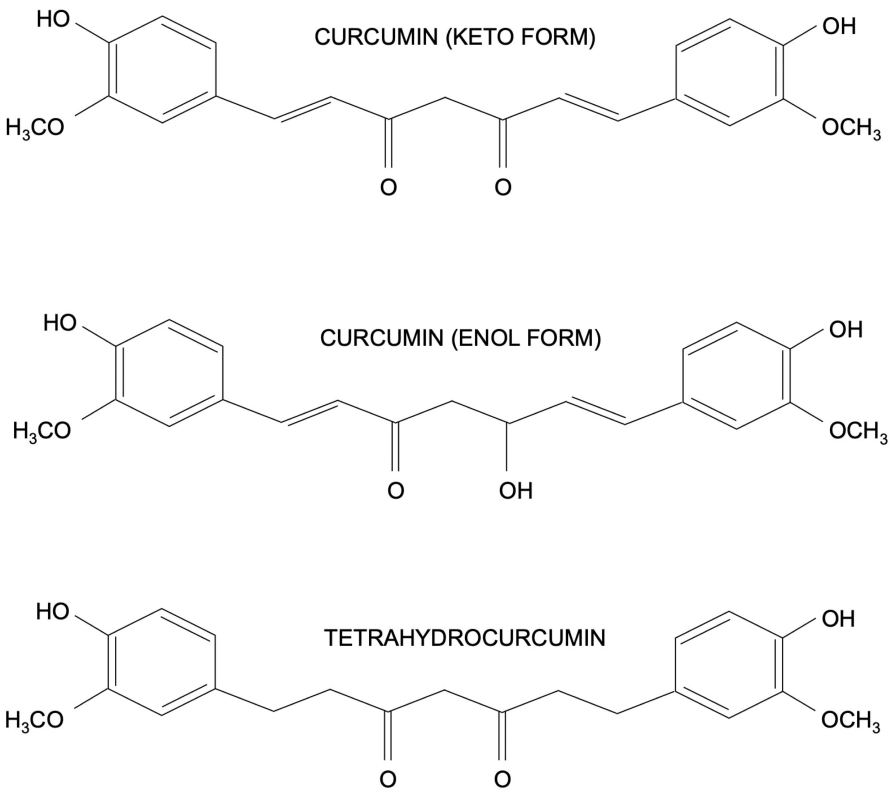

FIGURE 1 Chemical structure of the keto and enol forms of curcumin, and tetrahydrocurcumin. Curcumin has α, β-unsaturated carbonyl groups (*), but tetrahydrocurcumin lacks α, β dienes.

we measured messenger ribonucleic acid(mRNA) expression of several genes that are potentially involved in the pathogenesis of diabetic nephropathy.

2 | MATERIALS AND METHODS

All experiments were performed in accordance with approved protocols per the University of California, Irvine Institutional Animal Care and Use Committee (IACUC approval number AUP-19-015) consistent with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Data is reported per the recommendations in the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.1 | Experimental model

The diabetic chronic kidney disease (CKD) model in this study utilized uninephrectomy, high fat diet and one dose of streptozotocin which closely simulates human type 2 diabetic nephropathy.[25,26] Male Sprague–Dawley rats (body weight 225–250 g, Charles River) were subjected to right nephrectomy under inhaled isoflurane anesthesia. Buprenorphine 0.05 mg/kg was given via intramuscular injection for analgesia before the surgical incision was made. One week after surgery, CKD rats were placed on a high-fat diet (60% fat, TD.06414, Envigo/Teklad) for 4 weeks. After 3 weeks on high-fat diet, animals were fasted overnight for 12 h and then given a single intraperitoneal injection of streptozotocin (30 mg/kg, S0130, Sigma) to induce a mixed type 1 and type 2 DM. One week after streptozotocin injection, rats with fasting blood glucose of >200 mg/dL were considered diabetic and were randomly assigned to 5 experimental groups as described below. Animals were housed under a 12/12 h light/dark cycle and maintained at 22°C room temperature.

In the week prior to completion of the experiment, 24-h urine collections in metabolic cages (Teklad) and tail BP measurements (via tail-cuff plethysmography, CODA-S2 multi-channel, Kent Scientific) were performed. The reference controls (CTL) were age-matched healthy male rats. The experimental timeline is shown in Figure 2.

2.2 | THC, PPC and losartan treatment

Tetrahydrocurcumin was provided by Hub Therapeutics LLC and the lipid carrier PPC was purchased as PhosCol Liquid Concentrate (PHOSCON-16 where 1 teaspoon contains 3000 mg of purified PPC, Nutrasal Inc.). THC and PPC were administered via daily oral gavage at doses of 80 mg/kg[27] and 50 mg/kg, respectively. Losartan (124750–99-8 MFCD02092704; L0232-25G, Fisher Scientific) was dosed continuously in drinking water (180 mg/L).[28,29] Experimental CKD groups included reference cohorts with lipid carrier PPC or losartan alone, and were as follows: (1) untreated CKD, (2) PPC, (3) losartan, (4) THC + PPC, (5) THC + PPC + losartan. A deuterated form of THC was also tested, but did not increase blood THC levels measured 24 h after gavage dose as quantified by serum mass spectrometry and proteinuria reduction was similar to non-deuterated THC (data not shown).

2.3 | Tissue harvest

After 4 weeks on the assigned therapies, rats were euthanized by exsanguination via cardiac puncture under inhaled 5% isoflurane general anesthesia. Whole blood was used for hemoglobin assay at time of termination. Serum was aliquoted and kidney tissues were collected for histology and RNA analysis.

2.4 | Blood and urine biochemical assays

We measured hemoglobin by using an automated meter (Germaine Lab AimStrip Hemoglobin Test System, catalog# 23–111-280, Fisher Scientific). Blood urea nitrogen (BUN) and creatinine (Cr) were analyzed with colorimetric kits from BioAssay Systems, with 30x

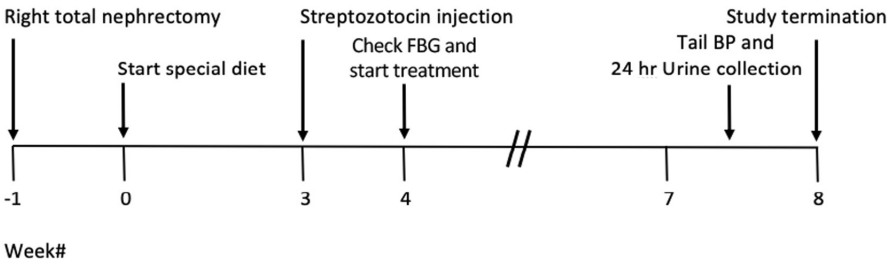

FIGURE 2 Experimental timeline in a diabetic single nephrectomy rat model. Right total nephrectomy was done 1 week before starting high fat special diet (60% fat) which was given for 4 weeks. After 3 weeks on special diet, animals received one dose of streptozotocin 30 mg/kg via intraperitoneal injection. At 1 week after the injection fasting blood glucose was checked and rats with values >200 mg/dL were randomized to treatment groups which included phosphatidylcholine (PPC) 50 mg/kg daily, losartan in drinking water (180 mg/L), PPC with tetrahydrocurcumin (THC) 80 mg/kg daily, or THC + PPC + losartan. Tail blood pressure (BP) measurement and 24-h urine collection were done prior to study termination after 4 weeks on treatment.

dilution used for urine creatinine. Urine albumin was measured with 1000x dilution using the Rat Albumin ELISA kit (ab108789, Abcam) and urine protein from 24-hour urine collection was measured using the Rat Urinary Protein Assay Kit (Chondrex Inc.). Creatinine clearance (mL/min*kg) was calculated as follows: [urine Cr×urine volume]/[serum Cr×1440×body weight]. Proteinuria per 24 h period was normalized to body weight and to creatinine clearance. Plasma levels of kidney injury molecule-1 (KIM-1) were measured with 4x dilution using a rat ELISA kit (ab223858, Abcam). Plasma fructosamine was measured via spectrophotometry at Phoenix Lab.

2.5 | TaqMan Real-Time PCR

Total mRNA was isolated from kidney samples using TRIzol® reagent (Invitrogen) per the manufacturer's protocol. First strand cDNA was made from 2 µg isolated total RNA primed with random hexamers using Superscript IV reverse transcriptase (Invitrogen). Gene transcript levels were quantified by Applied Biosystems TaqMan® gene expression assays using pre-made exon-spanning primers and TaqMan probe mixes (ThermoFisher Scientific). Reference assay ID for genes of interest were glutathione peroxidase (Gpx1, Rn00577994_g1), copper-zinc superoxide dismutase (CuZnSOD, Rn00566938_m1), transforming growth factor-β1 (TGF-β1, Rn00572010_m1), catalase (Rn00560930_m1), protein kinase C-α (PRKCA, Rn01496145_m1), type I collagen (col1a1, Rn01463848_m1), fibronectin-1 (Fn1, Rn00569575_m1), connective tissue growth factor (CTGF, Rn01537279_g1), hepatitis A virus cellular receptor 1 (Havcr1 a.k.a. KIM-1, Rn00597703_m1), heme oxygenase 1 (Hmox1, Rn00561387_m1), Kelch Like ECH Associated Protein 1 (Keap1, Rn00589292_m1), nuclear factor erythroid 2-related factor 2 (Nfe2l2, Mm01275375_m1), sirtuin 1 (Sirt1, Rn01428096_m1), SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic") family member 3 (Smad3, Rn00565331_m1), mitochondrial fission factor (MFF, Rn01400783_m1), dynamin like 1 (Dnm1l), also known as Dynamin-related protein 1 (Drp1, Rn00586466_m1), and mitochondrial fission 1 protein (Fis1, Rn01480911_m1).

Quantitative PCR was performed using the Mx3000P Detection System (Agilent Technologies), using standard cycler conditions (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Wells contained 25 uL PCR volume with 250 ng cDNA and 1X final concentrations of TaqMan® gene expression master mix and TaqMan® gene expression assay. The cDNA from a control kidney specimen was used to create the standard curves and calculate amplification efficiency. Threshold cycle (Ct) value and final quantitation values were expressed relative to GAPDH (Rn01775763_g1).

2.6 | Histopathological analysis

Paraffin sections of kidney were deparaffinized with xylene, dehydrated in alcohol series and stained with Masson's trichrome and periodic acid Schiff (PAS), then examined under a photomicroscope (Nikon Eclipse) by pathologists who were blinded to study groups (3–4 animals were studied from each group). Five images per animal were captured at 10X (for Masson's trichrome) or 40X (for PAS) objective. An ImageJ macro was used for quantification of kidney fibrosis (% area stained blue on Masson's trichrome) by an investigator blinded to the study groups.[24,30] In brief, the image is converted into a RGB stack and fibrosis is quantified by changing pixels that are blue (at 120% of red intensity) to have intensity value 1 and other pixels are set to 0. The 1 s are added up and expressed as fraction of total area.[30] Average fibrosis scores per CKD group were compared to CTL rats. The glomerular histopathology was graded into five categories: 0 (no sclerosis), 1 (less than 25% sclerosis), 2 (25%–50% sclerosis), 3 (50%–75% sclerosis), and 4 (75%–100% sclerosis). The glomerulosclerosis index score was calculated as follows: 1 x number of glomeruli with grade 1 + 2 × number of glomeruli with grade 2 + 3 × number of glomeruli with grade 3 + 4 × number of glomeruli with grade 4/total number of counted glomeruli. Approximately 5 glomeruli were examined in each animal. Tubulointerstitial fibrosis was graded on the basis of the extension of inflammatory cell infiltration, fibrosis, tubular dilatation, and atrophy as follows: 0 (normal), grade 1 (<25%), grade 2 (25%–50%), grade 3 (50%–75%), and grade 4 (75%–100%).[31]

2.7 | Statistical analysis

Data were screened for outliers using Grubbs' test (extreme studentized deviate method, http://graphpad.com/quickcalcs/grubbs1/). Bartlett's test was used to assess homogeneity of variances across groups. For datasets with equal variances, group data were analyzed using one-way Analysis of Variance (ANOVA) with post hoc Tukey, and $p < 0.05$ was considered significant. For nonparametric data, Kruskal-Wallis analysis was used ($p < 0.05$ considered significant) with Dunn's Multiple Comparison Test. Data are reported as mean ± standard error. Figures were generated using GraphPad Prism 4 software (GraphPad Software).

3 | RESULTS

3.1 | General data

Induction of DM, that is, fasting blood glucose >200 mg/dL, was successful in 83% of the rats. Fasting blood glucose averaged 98 ± 6.8 mg/dL in control animals versus 478 ± 0.6 mg/dL in diabetic single nephrectomy rats at time of randomization to CKD treatment groups. There was one death in the THC + PPC + Losartan group. At study end, there were 6–9 animals per experimental group (Table 1).

Diabetic CKD animals had significantly lower body weight at study termination, compared to controls. Systolic BP was significantly lower in the THC + PPC + losartan group compared to untreated CKD rats; there was a trend for lower BP in the losartan group which did not reach statistical significance. Hemoglobin,

TABLE 1 General data from the 6 study groups.

	CTL ($n = 6$)	CKD ($n = 8$)	PPC ($n = 6$)	Losartan ($n = 8$)	THC + PPC ($n = 9$)	THC + PPC + Losartan ($n = 8$)
Baseline fasting blood glucose (mg/dL)	105 ± 1	383 ± 45*	412 ± 60*	404 ± 48**	468 ± 37**	407 ± 35**
Systolic blood pressure (mmHg)	115.1 ± 10.6	129.0 ± 6.7	117.1 ± 8.2	109.6 ± 5.2	126.4 ± 6.1	99.2 ± 6.1#
Hemoglobin (g/dL)	13.6 ± 0.2	13.8 ± 0.1	13.5 ± 0.2	13.6 ± 0.4	13.4 ± 0.1	13.7 ± 0.2
Body weight at termination (g)	522.0 ± 6.9	382.5 ± 24.1***	377.8 ± 13.6**	356.3 ± 8.4***	399.7 ± 24.3**	399.1 ± 21.8**
Heart/body weight ratio (g/kg)	2.6 ± 0.1	2.9 ± 0.2	2.7 ± 0.2	2.8 ± 0.06	2.7 ± 0.07	2.5 ± 0.01
Fructosamine at termination (umol/L)	170 ± 4	268 ± 8**	266 ± 25**	N/A	280 ± 18***	250 ± 36*
Blood urea nitrogen (mg/dL)	16.1 ± 1.3	29.2 ± 2.5	33.8 ± 4.8	34.8 ± 3.1*	32.4 ± 2.8	36.0 ± 6.0*
Serum creatinine (mg/dL)	0.17 ± 0.007	0.22 ± 0.019	0.23 ± 0.008	0.24 ± 0.02	0.21 ± 0.01	0.21 ± 0.2
Urine albumin/creatinine ratio (mg/g)	23 ± 9.6	664 ± 197*	717 ± 244*	531 ± 159	211 ± 35	351 ± 146
24 h urine creatinine clearance (mL/min*kg)	14.7 ± 1.0	3.6 ± 0.2***	6.4 ± 1.4*	4.5 ± 1.4***	7.4 ± 1.2*	5.5 ± 1.7**
24 h urine volume (mL)	16.6 ± 3.5	96.3 ± 19.1*	72.7 ± 20.3	74.5 ± 15.6	89.6 ± 12.4*	77.1 ± 18.6
Urine protein/creatinine ratio (mg/g)	7.5 ± 0.7	59.6 ± 11.4**	62.5 ± 11.5**	56.9 ± 10.8**	43.8 ± 6.0	44.2 ± 8.8
24 h proteinuria per body weight (mg per 100 g)	0.26 ± 0.02	0.70 ± 0.13*	0.61 ± 0.13	0.58 ± 0.09	0.66 ± 0.1	0.55 ± 0.1
24 h proteinuria per creatinine clearance (mg per mL/min*kg)	9.9 ± 1.0	68.2 ± 7.6**	56.9 ± 12.2*	67.1 ± 11.8**	54.0 ± 7.0*	61.15 ± 10.5**

Abbreviations: CKD, chronic kidney disease (diabetic single nephrectomy rat model); CTL, controls; PPC, polyenylphosphatidylcholine; THC, tetrahydrocurcumin. Data are reported as mean ± standard error.

*$p < 0.05$ vs. CTL; **$p < 0.01$ vs. CTL; ***$p < 0.001$ vs. CTL.

#$p < 0.05$ vs. CKD.

serum creatinine and heart weight adjusted to body weight (a marker for cardiac hypertrophy) were not different between groups. At study termination, plasma fructosamine, a marker of glycemic control over the previous 2–3 weeks, was not significantly different between the CKD groups (not tested in losartan group). Plasma KIM-1 was elevated across CKD groups except in the THC + PPC + losartan group where KIM-1 was not significantly different from CTL animals.

Urine 24-hour creatinine clearance was significantly decreased, and albuminuria was increased in CKD animals (3.6 ± 0.2 mL/min*kg and 664 ± 197 mg albumin per gram urine creatinine respectively, versus 14.7 ± 1.0 mL/min*kg and 23 ± 9.6 mg/g creatinine in control (CTL) rats). The THC + PPC treatment group showed an improvement in creatinine clearance, approximately double the average creatinine clearance of untreated CKD rats. The THC + PPC and THC + PPC + losartan groups demonstrated the greatest effect on lowering albuminuria (Figure 3).

3.2 | Quantitative PCR

Total mRNA analysis of genes important in oxidative stress and fibrosis pathways showed that combination THC + PPC + losartan therapy significantly decreased PRKCA, KIM-1 (encoded by the gene Havcr1), Hmox1 and type I collagen, and increased CuZnSOD in the kidneys of diabetic CKD rats (Figure 4). TGF-β1, Fis1, Fn1, catalase and Sirt1 mRNA levels were significantly increased in CKD rats, while levels were similar to CTL animals in all the treatment groups studied. No significant differences were observed in the other factors analyzed (Figure 4).

3.3 | Fibrosis scores on histology

The area of fibrosis noted on Masson's trichrome staining was increased 8-fold in untreated diabetic CKD animals compared to CTL rats. Fibrosis was less severe in the PPC group (3-fold increase in area compared to CTL) and in the losartan and THC + PPC + losartan groups (2-fold increase in area compared to CTL). Therapy with THC + PPC demonstrated the greatest protection against kidney fibrosis, with fibrosis scores similar to CTL (Figure 5). On PAS stain, glomerulosclerosis was not different between treatment groups, and tubulointerstitial injury was significantly decreased with THC + PPC + losartan compared to untreated diabetic CKD rats (Figure 5).

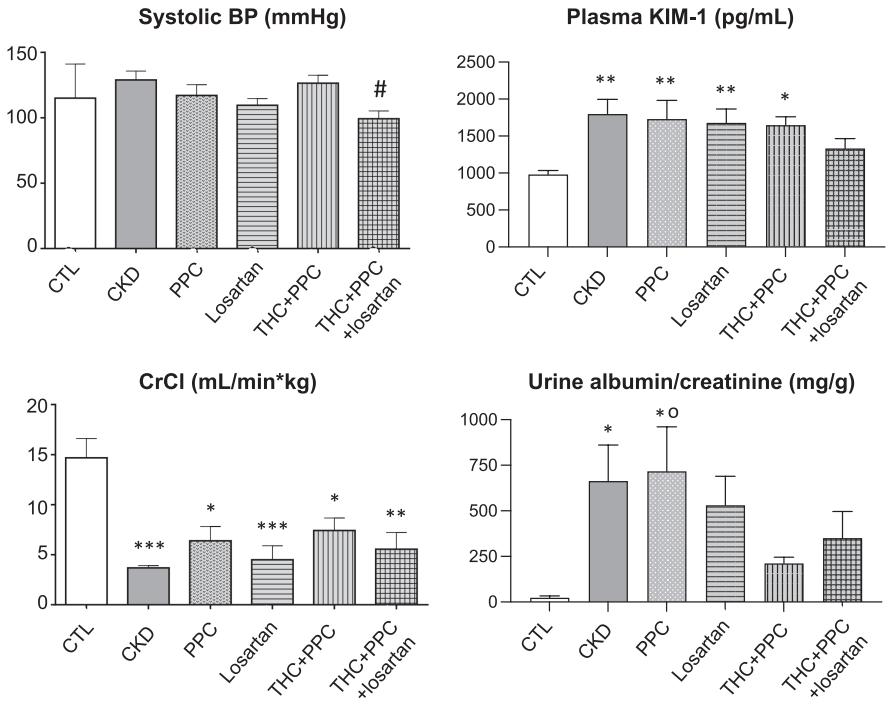

FIGURE 3 Effects of THC and PPC lipid carrier with/without losartan in diabetic CKD rats. BP, blood pressure; CKD, chronic kidney disease; CrCl, creatinine clearance; CTL, control animals; KIM-1, kidney injury molecule-1; PPC, polyenylphosphatidylcholine; THC, tetrahydrocurcumin. $^{*}p<0.05$ vs. CTL, $^{**}p<0.01$ vs. CTL, $^{***}p<0.001$ vs. CTL; $\#p<0.05$ vs. CKD; o $p<0.05$ vs. THC + PPC.

4 | DISCUSSION

In this model of diabetic nephropathy induced by a combination of uninephrectomy, streptozotocin and high-fat diet, CKD rats manifested weight loss, polyuria, proteinuria and decreased creatinine clearance. We noted that THC + PPC alone has potential therapeutic benefits, whereby kidney fibrosis was significantly decreased on histology and kidney mRNA levels of KIM-1 and col1a1 were decreased compared to untreated CKD animals. However, the addition of THC + PPC to losartan derived the most benefit in terms of significantly lowering BP concurrent with increased kidney mRNA levels of CuZnSOD and decreased PRKCA, KIM-1 and type I collagen. There was a trend toward decreased albuminuria and plasma KIM-1 levels, and improved creatinine clearance. These changes were independent of glycemic status as elevated plasma fructosamine was similar across CKD groups.

Though not statistically significant, losartan therapy was associated with a trend for decreased systolic BP (average 109.6 mmHg) and albuminuria (average urine albumin 531 mg per gram creatinine) compared to untreated CKD animals (average values 129 mmHg and 664 mg/g respectively). The animals were receiving a losartan dose of 40–50 mg/kg/day (losartan 180 mg/L in drinking water, water intake 80–100 mL/day in diabetic animals with average body weight 356 g) and doses of 20–25 mg/kg/day were previously reported to decrease BP and/or proteinuria.[32–35] One important factor is timing of initiation of losartan therapy, as there is a time-dependent increase in albuminuria in animal DM models,[36] as well as duration of therapy (losartan was given up to 18 weeks in one study[33]). Further, the majority of prior investigations were in type 1 DM models where a high dose of streptozotocin was utilized (50–55 mg/kg).[33,35] To our knowledge, our study is the first to test losartan in the uninephrectomy, high fat diet and low-dose streptozotocin rat model which closely replicates human type 2 diabetic nephropathy.[25,26] Of note, there are conflicting reports from others whereby losartan therapy did not lower BP in a rat type 1 DM model[37] nor in transgenic Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats.[34] Thus, the anti-proteinuric effects of RAAS blockade such as lowering of TGF-β1 as observed in the current study, may precede or occur independent of BP lowering. Given the short half-life of losartan (2 h), future studies should consider twice-daily dosing versus testing newer angiotensin receptor blockers that have a longer duration of action.[38]

In the current investigation, PPC was used as lipid carrier to increase THC bioavailability. Marczylo et al. reported five-fold increase in curcumin plasma levels when dosed with PPC in rats.[16] We previously studied THC + PPC in 5/6 nephrectomized rats and reported improved expression of antioxidant enzymes in the remnant kidney, with decreased renal fibrosis, proteinuria and hypertension.[24] The current diabetic nephropathy study showed that PPC administration alone resulted in significant effects on the histologic tubulointerstitial score, as well as Hmox1 and Kim1 (Hvacr1) mRNA levels (Figures 4 and 5). Therefore, the favorable effects observed in the THC treatment groups are attributable in part to PPC. Further determination of THC-specific benefits in vivo may be challenging given the requirement for THC to be delivered with a carrier to optimize bioavailability.[9]

In our prior study in 5/6 nephrectomized animals, 1% THC was mixed into chow which resulted in much higher drug exposure (1000 mg/kg, assuming diet intake of 25 g per day with average rat weight 250 g in that study).[24] Our current investigation utilized a THC dose of 80 mg/kg which other groups have reported in diabetic rat studies[15,27]; it is possible that a higher dose is needed to significantly modulate proteinuria.

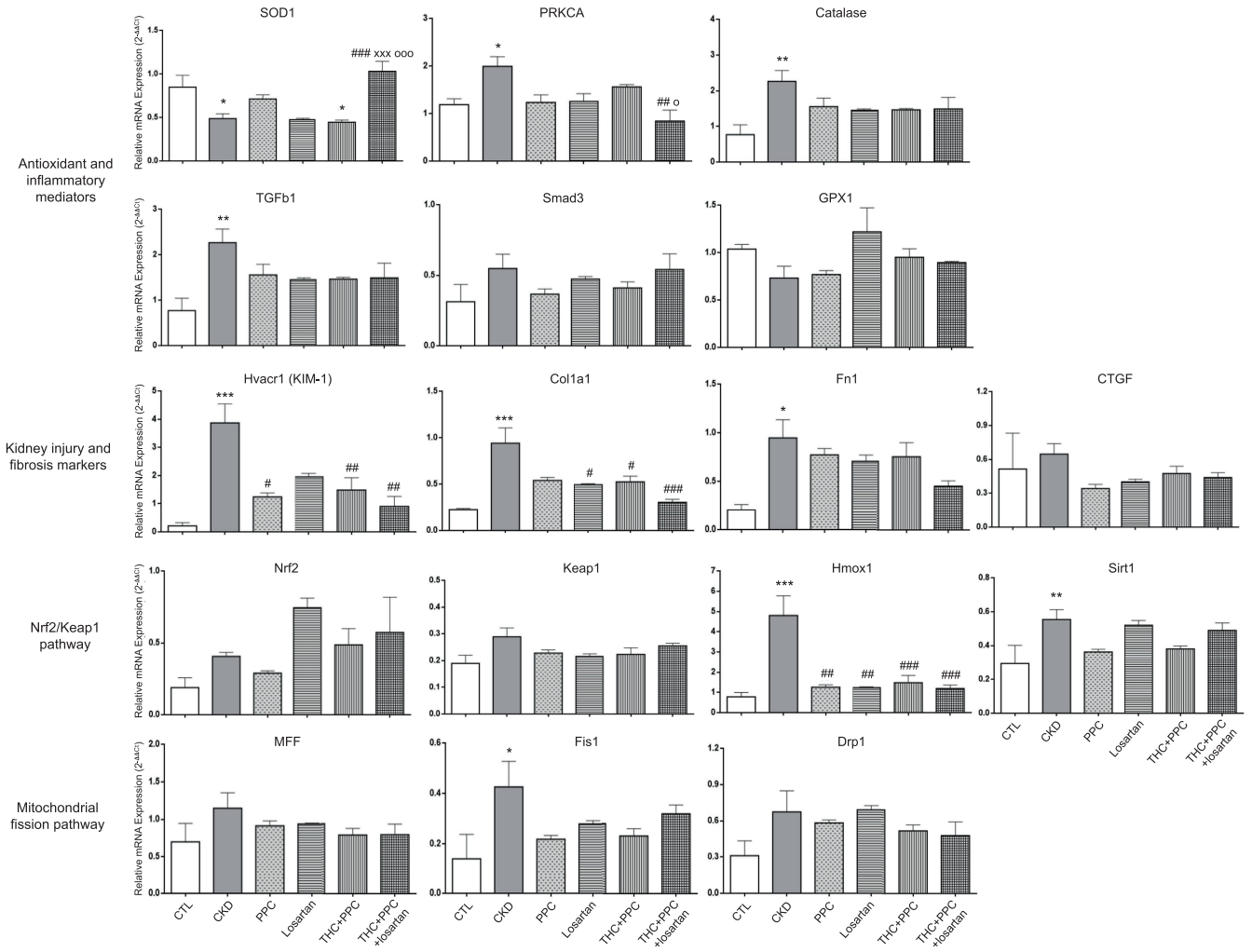

FIGURE 4 Kidney mRNA expression of oxidative stress and fibrosis genes in diabetic chronic kidney disease (CKD) rats, normalized to GAPDH. Therapy with tetrahydrocurcumin (THC), lipid carrier polyenylphosphatidylcholine (PPC) and angiotensin-II-receptor blocker losartan significantly decreased protein kinase C-α (PRKCA), kidney injury molecule-1 (KIM-1, encoded by the gene Hvacr1), heme oxygenase 1 (Hmox1) and type I collagen (col1a1) compared to untreated CKD animals, whereas copper-zinc superoxide dismutase (CuZnSOD) was significantly increased. Transforming growth factor-b1 (TGF-b1), mitochondrial fission 1 protein (Fis1), fibronectin-1 (Fn1), catalase and sirtuin 1 (Sirt1) mRNA levels were significantly increased in diabetic CKD rats compared to control (CTL) animals; levels of these inflammatory/ oxidative stress markers in the treatment groups were equivalent to CTL but not significantly decreased compared to CKD rats. The remaining factors studied were not significantly different between experimental groups: glutathione peroxidase-1 (Gpx1), connective tissue growth factor (CTGF), Kelch Like ECH Associated Protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nfe2l2 also known as Nrf2), SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic") family member 3 (Smad3), mitochondrial fission factor (MFF), dynamin like 1 (Dnm1l). *$p < 0.05$ vs. CTL, **$p < 0.01$ vs. CTL, ***$p < 0.001$ vs. CTL; #$p < 0.05$ vs. CKD, ##$p < 0.01$ vs. CKD, ###$p < 0.001$ vs. CKD; xxx $p < 0.001$ vs. Losartan; o $p < 0.05$ vs. THC + PPC, ooo $p < 0.001$ vs. THC + PPC.

Deficiency of superoxide dismutase plays a major role in the pathophysiology of diabetic nephropathy. Cytosolic CuZnSOD (SOD1) and extracellular CuZnSOD (SOD3) are downregulated in the kidneys of KK/Ta-Akita mice which exhibit progressive diabetic nephropathy, and treatment with the SOD mimetic, tempol, ameliorated nephropathy without altering blood glucose levels.[39] In the current study, only the combination THC/PPC/losartan therapy significantly increased kidney CuZnSOD mRNA expression with a trend for further lowering of albuminuria compared to the losartan-only group. It is possible that more significant benefits in terms of preserving creatinine clearance and decreasing proteinuria may emerge with longer duration of therapy beyond 4 weeks. We did not observe upregulation of a different antioxidant, catalase, with THC treatment.

The combination of THC, PPC, and losartan significantly decreased kidney mRNA levels of PRKCA, consistent with prior reports that the parent compound curcumin attenuated nephropathy in rats with type 1 DM via inhibition of PRKCA and -β1.[40] A central role for PRKCA in diabetic nephropathy was demonstrated in knockout models whereby diabetic PRKCA−/− mice were resistant to vascular endothelial growth factor (VEGF)-induced albuminuria and glomerular hyperfiltration.[41] In tandem with increased CuZnSOD and

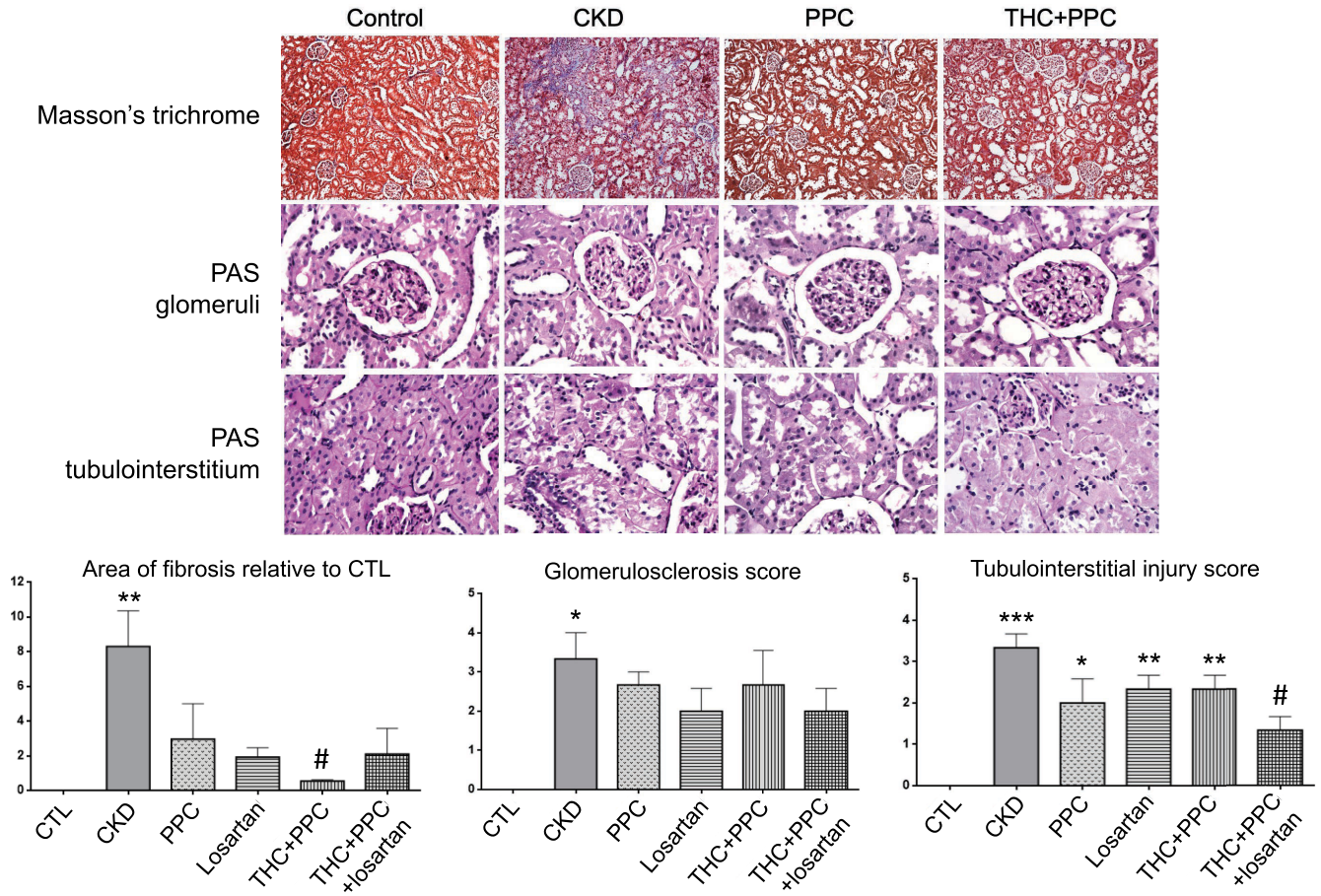

FIGURE 5 Representative micrographs of kidney tissue stained with Masson's trichrome to assess degree of fibrosis (percent area stained blue, 10X objective), and periodic acid Schiff (PAS) to assess glomerulosclerosis and tubulointerstitial injury (40X objective) relative to control (CTL) rats. Fibrosis was increased 8-fold in diabetic chronic kidney disease (CKD) and was significantly attenuated with tetrahydrocurcumin (THC) and PPC (polyenylphosphatidylcholine) therapy. Glomerulosclerosis was not different between groups, and tubulointerstitial injury was significantly decreased in diabetic CKD rats treated with THC + PPC + losartan. $^{*}p < 0.05$ vs. CTL; $^{**}p < 0.01$ vs. CTL; $^{***}p < 0.001$ vs. CTL; $\#p < 0.05$ vs. CKD.

decreased PRKCA, the kidney injury marker KIM-1 was decreased, and fibrosis was ameliorated on histology (Figure 5) as well as on molecular analysis (decreased col1a1, Figure 4). Fn1, an extracellular matrix protein which accumulates in the glomerular mesangium in diabetic nephropathy,[42] was significantly elevated in untreated CKD rats and trended lower in the treatment groups, though this trend did not reach statistical significance (Figure 4). CTGF, another extracellular matrix protein that has been implicated in diabetic glomerulosclerosis,[43] was not modulated by THC.

TGF-β1 exerts several effects in the pathogenesis of diabetic nephropathy ranging from increasing glomerular permeability and promoting fibrosis[44]; in the current study, all the agents studied (THC, PPC, losartan) were beneficial in decreasing kidney TGF-β1 levels compared to untreated CKD rats. In our prior report, THC treatment in 5/6 nephrectomized rats significantly increased renal Gpx1[24] which is an antioxidant (scavenging) factor. In the current study, there was a trend for increased Gpx1 with THC treatment in diabetic CKD which did not reach statistical significance. However, Gpx1 deficiency appears to be less critical in the pathogenesis of diabetic nephropathy than loss of CuZnSOD; de Haan and colleagues previously reported that Gpx1−/− mice exhibited equivalent degree of renal injury as wildtype mice in a streptozotocin-induced diabetic nephropathy model.[45] Smad signaling pathways have been implicated in TGF-β1 upregulation in diabetic nephropathy[46]; however, we did not detect inter-group differences in Smad3 in our study (Figure 4). In the current study, mitochondrial fission (MFF, Fis1 and Drp1),[47,48] Nrf2/Keap1 and autophagy (Sirt1)[49] pathways were not significantly involved in the renoprotective mechanisms of THC. The negative Nrf2/Keap1 findings are consistent with prior work that has shown THC to be a weak inducer of Nrf2 translocation to the nucleus.[24,50]

Compared with the 5/6 nephrectomy model where THC reduced cardiac hypertrophy,[24] there was a trend for decreased heart weight/body weight ratio with THC + PPC + losartan treatment in the diabetic rats (2.5 ± 0.01 versus 2.9 ± 0.2 g/kg in untreated CKD animals) which did not reach statistical significance. This may be partly explained by lower average BP in the diabetic CKD animals compared to 5/6 nephrectomized rats (129/88 versus 140/94 mmHg[24]).

In summary, addition of THC with the lipid carrier PPC to losartan therapy significantly improved antioxidant CuZnSOD mRNA levels and decreased PRKCA, KIM-1 and type I collagen in the kidneys of diabetic rats, and induced further lowering of BP and albuminuria. In particular, CuZnSOD expression was markedly increased compared to the losartan-only group. Clinical trials are needed to examine these effects in diabetic patients and to clarify potential benefits on slowing CKD progression.

AUTHOR CONTRIBUTIONS

Khazaeli, Mahyar: Project assistant, drafting the manuscript; Nunes, Ane C.F: Data analysis and project assistant; Zhao, Yitong: Data analysis; Khazaeli, Mahziar: drafting the manuscript; Prudente, John: Project Assistant; Vaziri Nosratola D: Mentoring the project; Singh, Bhupinder: Mentoring the project; Lau, Wei Ling: Mentoring the project, data analysis, drafting the manuscript.

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DATA AVAILABILITY STATEMENT

Data is reported per the recommendations in the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLOSURES

The authors declare that they have no relevant or material financial interests that relate to the research described in this paper.

ETHICS STATEMENT

All experiments were performed in accordance with approved protocols per the University of California, Irvine Institutional Animal Care and Use Committee (IACUC protocol# AUP-19-015) consistent with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

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